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Description

The present invention relates to human Tumor Necrosis Factor (TNF) Binding Protein I, herein designated TBP-I (= soluble Tumor Necrosis Factor Receptor type I (sTNF-RI)), and more particularly, to the cloning of the gene coding for said protein and its expression in eukaryotic host cells.

TNF-α and TNF-β (lymphotoxin) are structurally related polypeptide cytokines, produced primarily by mononuclear leukocytes, whose effects on cell function constitute a major factor in the elicitation of the inflammatory response. The TNFs affect cells in different ways, some of which resemble the functional modes of other inflammatory mediators, like interleukin-1 (IL-1) and interleukin-6 (IL-6). What appears most distinctive regarding the activity of the TNFs is that many of their effects can result in cell and tissue destruction. Increasing evidence that over-induction of those destructive activities contributes to the pathogenesis of a number of diseases, makes it of particular interest to elucidate their mechanisms and the ways they are regulated (Old, L.J. (1988) Sci.Am. 258, pp. 41-49).

High affinity receptors, to which both TNF-α and TNF-β bind (Beutler, B.A., et al. (1985) J.Exp.Med. 161, pp. 984-995) play a key role in the initiation and in the control of the cellular response to those cytokines. These receptors are expressed on the surfaces of a variety of different cells. Studies showing that antibodies reacting with their extracellular portions affect cells in a manner very similar to the TNFs, demonstrate that the receptors and cellular components associated with them are sufficient to provide the intracellular signalling for the effects of the TNFs (Espevik, T., et al., (1990) J.Exp.Med. 171, pp. 415-426).

Other studies have shown that molecules related to the TNF receptors (TNF-Rs) exist also in soluble forms. Two immunologically distinct species of such soluble TNF-Rs, designated TNF Binding Proteins I and II, or TBP-I and TBP-II, respectively, were recently isolated from human urine (Engelmann, H., et al., (1989) J.Biol.Chem. 264, pp. 11974-11980; Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536; Olsson, I., et al., (1989) Eur.J.Haematol. 42, pp. 270-275; Seckinger, P., et al., (1989a) J.Biol.Chem. 264, pp. 11966-11973). Immunological evidence indicated that the two proteins are structurally related to two molecular species of the cell surface TNF-R (the type I and type II receptors, respectively). Antibodies to each of the two soluble proteins were shown to block specifically the binding of TNF to one of the two receptors and could be used to immunoprecipitate the receptors. Antibodies against one of the two soluble proteins (TBP-I) were also found to induce effects characteristic of TNF in cells which express the immunologically cross-reactive cell receptors (Engelmann, H., et al., (1990) ibid.). Like the cell surface receptors for TNF, the soluble forms of these receptors specifically bind TNF and can thus interfere with its binding to cells. It was suggested that they function as physiological inhibitors of TNF activity (Engelmann et al., 1989 (ibid.); Olsson et al., 1989 (ibid.); Seckinger et al., 1989a (ibid.)).

Soluble forms of cell surface receptors may be derived from the cell surface form of the receptor by proteolytic cleavage, or by a different mechanism proposed in two recent studies describing the cloning of the cDNAs for the receptors to IL-4 and IL-7. Besides cDNA clones encoding the full length receptors, clones which encode truncated, soluble forms of these receptors were also isolated in these studies. It was suggested that these latter clones are derived from transcripts specifically encoding soluble forms of the receptors, transcribed from the same genes which encode the cell surface forms, but differently spliced (Mosley, B., et al., (1989) Cell 59, pp. 335-348; Goodwin, R.G., et al., (1990) Cell 60, pp. 941-951).

Two recent studies have described the molecular cloning and expression of human type I TNF cell surface receptor (Loetscher, H., et al. (1990) Cell 61, pp. 351-359; Schall, T.J., et al., (1990) Cell 61, pp. 361-370).

The present invention relates to the production or human TBP-I by a method comprising transfection of eukaryotic, preferably CHO cells, with an expression vector comprising a DNA molecule encoding the whole type I human TNF receptor (TNF-RI), culturing the transfected eukaryotic cells and isolating said TBP-I from the medium When said whole DNA molecule is used, soluble proteins are produced by the transfected cells, along with the cell surface receptor, and are secreted into the medium.

Figure 1 shows the nucleotide sequence of the type I TNF receptor cDNA and the predicted amino acid sequence of the encoded protein.

(A) shows the probes used for screening for the cDNA, wherein:

(a) shows the NH_2-terminal amino acid sequence of TBP-I;

(b) shows synthetic oligonucleotide probes, designed on the basis of the NH_2-t rminal amino acid sequence, used for initial screening; and

(c) and (d) are probes overlapping with (b), used to confirm the validity of clones isolated in the initial screening.

(B) is the schematic presentation of the cDNA clones isolated from a human colon (C2) and from CEM-lymphocyt s (E13) librari s and a diagram of the complete cDNA structure. Untranslated sequences are

represented by a line. Coding regions are boxed. The shaded portions represent th sequences which encode the signal peptide and the transmembrane domains.

(C) shows the hydropathy profile of the predicted amino acid sequence of the TNF receptor. Hydrophobicity (above the line) and hydrophilicity (below the line) values were determined using the sequence analysis software package of the University of Wisconsin genetic computer group (UWCG) according to Kyte and Doolittle (1982). The curve is the average of the hydropathy index for each residue over a window of nine residues.

(D) depicts the nucleotide and predicted amino acid sequences of the type I TNF receptor. The presumptive start and stop signals are denoted by asterisks; the three sequences derived from TBP-I by broken overlining; the transmembrane and leader domains by round-ended boxes; and the four repetitive sequences in the extracellular domain by thick underlining. Cysteine residues are boxed. Glycosylation sites are overlined and the presumptive polyadenylation signal is underlined.

Figure 2 shows the detection of type I TNF-R using monoclonal antibodies to TBP-I in CHO cells transfected with E13 cDNA. CHO cells, clones R-18 (transfected with an expression vector in which the E13 cDNA was placed under the control of an SV40 promoter) and C-6 (control; a clone of cells transfected with an expression vector in which E13 was placed in the inverse orientation), and HeLa cells, were stained with the anti-TBP-I monoclonal antibodies 17, 18, 20 and 30 followed by incubation with FITC conjugated anti-mouse F(ab). Fluorescence intensity was compared with that observed when a mouse monoclonal antibody against TNF was used in the first step of the staining as a control.

Figure 3 shows reversed phase HPLC of the CHO-produced, soluble form of the type I TNF-R.

A concentrate of the conditioned medium of the CHO R-18 clones (see Fig. 2) and a concentrate of the CHO C-6 clone to which 3 ng pure TBP-I was added, were applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (---). Fractions were examined for content of protein (—) and of the soluble form of the type I receptor by an ELISA (▬), (as described in Example 1: Procedures). None of the eluted fractions of a concentrate of the CHO C-6 clone, without addition of TBP-I, was found to contain any detectable amounts of the soluble form of the receptor (not shown).

Figure 4 shows the time course of the release of COOH-terminal amino acids from TBP-I by carboxypeptidase Y.

Figure 5 shows the construction of plasmid pSV-TBP, which contains the DNA sequence encoding TBP-I fused to the strong SV40 early gene promoter.

Figure 6 shows the construction of the plasmid pCMV-TBP, which contains the DNA sequence encoding TBP-I fused to the cytomegalovirus (CMV) promoter.

Purified TBP-I isolated from human urine was described in European Patent Application EP 0 308 378 of the present applicants and shown to contain at the N-terminus the amino acid sequence shown in Fig. 1Aa.

The COOH-terminal of TBP-I was determined now and shown to contain a major fraction containing the sequence Ile-Glu-Asn denoted by broken overlining at positions 178-180 in Fig. 1D, and at least one minor fraction including at least two further amino acids Val-Lys at positions 181-182.

The invention relates to a method for the production of human Tumor Necrosis Factor Binding Protein I (TBP-I), biologically active precursors and analogs thereof, which comprises:

i) transfecting eukaryotic cells with an expression vector comprising a DNA molecule encoding the whole type I human TNF receptor, and

ii) culturing the transfected cells, whereby the desired protein is produced and secreted into the medium.

The DNA sequence encoding the whole type I TNF receptor is depicted in Figure 1D. The soluble domain thereof includes the sequence down to position 180 (Asn) or 182 (Lys) or even some additional amino acids after position 182.

The human TBP-I proteins produced by the transfected cells according to the method of the invention and secreted into the medium may have at the N-terminus the sequence Asp-Ser-Val denoted by broken overlining at positions 20-23 in Fig. 1D (TBP-I), or the sequence Leu-Val-Pro at positions 9-11 or Ile-Tyr-Pro at positions 1-3 or any other sequence between Ile(+1) and Asp(20). The proteins may have it the COOH terminal any of the sequences described above. All these human TBP-I proteins, if biologically active with TBP-I-like activity, are encompass d by th invention as precursors and analogs of TBP-I.

According to the invention, oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBP-I, were synthesized by known methods and used for screening for the cDNA coding for TBP-I in cDNA libraries. In a human colon cDNA library, a C2 cDNA insert was found which hybridized to said probes and it was used for further screening in a human CEM-lymphocytes lambda ZAP cDNA library, thus leading to the cDNA shown in Fig. 1D.

The DNAs of positive clones w re then inserted into appropriately constructed expression vectors by techniques well known in the art. In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. The gene must be preceded by a promoter in order to be transcribed There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

The DNA molecule comprising the nucleotide sequence coding for a protein comprising the amino acid sequence of TBP-I, i.e. TBP-I, a precursor or an analog thereof, preceded by a nucleotide sequence of a signal peptide and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector.

In a preferred embodiment, the introduced DNA molecule will he incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) nay be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

The eukaryotic host cells are transfected according to the invention with plasmids comprising the cDNA encoding the whole type I TNF receptor. Preferred eukaryotic hosts are mammalian cells, e.g., human, monkey, mouse and chinese hamster ovary (CHO) cells. They provide the soluble form of the protein, besides the cell surface receptor, and provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Preferred mammalian cells according to the invention are CHO cells.

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired soluble protein, that is secreted into the medium, and may then be isolated and purified by any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

In a preferred embodiment, CHO cells are transfected with the type I TNF-R cDNA shown in Fig. 1D and they produce both the cell surface receptor and TBP-I, its soluble form, and/or precursors and analogs thereof.

The data presented in the present application are consistent with the notion that TBP-I - the soluble form for the type I TNF-R - constitutes a fragment of the cell surface form of this receptor, corresponding to its extracellular domain. The receptor is recognized by several monoclonal antibodies to TBP-I which interact with several spatially distinct epitopes in this protein. The amino acid sequence in the extracellular domain matches the sequence of TBP-I.

Particularly informative with regard to the mechanism of formation of TBP-I is the finding described in the present application, that a soluble form of the type I TNF-R is produced by CHO cells which were transfected with the TNF-R cDNA. This implies that cells possess some mechanism(s) which allow(s) the formation of the soluble form of the TNF-R from that same transcript that encodes the cell surface form.

The low rate of production of the soluble form of the type I TNF-R by the E13-transfected CHO cells does not necessarily reflect maximal activity. In HT29 cells, the spontaneous release of a soluble form of type I TNF-R occurs at about a 10-fold higher rate than that observed with the CHO-R-18 clone.

A likely mechanism whereby soluble forms of TNF receptors can be derived from the same transcripts which encode the cell surface forms is proteolytic cleavage. Indeed, flanking the amino acid residue which corresponds to the NH_2-terminus of TBP-I there are, within the sequence of amino acids of the receptor, two basic amino acid residues (Lys-Arg) which can serve as a site of cleavage by trypsin-like proteases. The identity of the proteases which might cause cleavage to take place at the COOH terminus of TBP-I is not known.

The invention will be illustrated by the following examples:

EXAMPLE 1: PROCEDURES

a) Determination of amino acid sequences within the TNF-binding proteins TBP-I and TBP-II

The TNF Binding Proteins TBP-I and TBP-II were isolated from concentrated preparations of urinary proteins, as described previously (Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536) by ligand (TNF) affinity chromatography followed by reversed phase HPLC. TBP-I was cleaved with cyanogen bromide, yielding two peptides which, following reduction and alkylation, were isolated by reversed phase HPLC. The two peptides (CNBr-1 and CNBr-2 in Table I) were subjected to NH_2-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (Model 475A, Applied Biosystems Inc., Foster City CA). The sequence found for one of the peptides (CNBr-1) was identical to the NH_2 sequence of the intact TBP-I protein.

The COOH terminal sequence of amino acids in TBP-I was determined by digestion of the protein with carboxypeptidase Y followed by sequential analysis of the released amino acids. A sample of pure TBP-I (32μg) was mixed with 1 nmole of norleucine, as an internal standard, dried thoroughly and resuspended in 8 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.8 μg carboxypeptidase Y (Sigma, St. Louis, MO). Digestion was performed at room temperature. 2 μl Aliquots withdrawn at various time points were acidified by adding 3 μl of 10% acetic acid to each, followed by addition of 15 μl 0.5% EDTA. They were then subjected to automated amino acid analysis (Applied Biosystems, U.K. model 420A). The results (shown in Fig. 4) indicate the sequence -Ile-Glu-Asn-COOH. Minor fractions were detected containing two or more additional amino acids.

Sequences within TBP-II were determined by generation of tryptic peptides of the protein. A sample of pure TBP-II (200 μg) was reduced, alkylated and repurified on an Aquapore RP-300 reversed-phase HPLC column. Fractions containing the modified protein were pooled and the pH was adjusted to 8.0 with $NaHCO_3$. Digestion with TPCK-trypsin (238 U/mg, Millipore Corp.,,Freehold, NJ) was performed for 16 h. at room temperature at an enzyme to substrate ratio of 1:20 (w/w). The digest was loaded on a C_{10} RP-P reversed phase HPLC column (Synchrom, Linden, IN) and the peptides separated by a linear 0 to 40% acetonitrile gradient in 0.3% aqueous trifluoroacetic acid. The NH_2 terminal amino acid sequences of the peptides and of the intact protein (N-terminus) are presented in Table I. The peptides were numbered according to their sequences of elution from the RP-P column. In the fractions denoted as 39,44,46,53 and 54, where heterogeneity of sequences was observed, both the major and the secondary sequences are presented.

b) Isolation of cDNA clones

Three mixtures of synthetic oligonucleotide probes (Figs. 1Ab, 1Ac) generated from the nucleotide sequence deduced from the NH_2-terminal amino acid sequence of TBP-I (Fig. 1Aa) were used for the screening of cDNA libraries. Initial screenings were carried out with 48-fold degenerated, 26-mers into which deoxyinosine was introduced, wherever the codon ambiguity allowed for all four nucleotides (Fig. 1Ab). The validity of positive clones was examined by testing their hybridization to two mixed 17-mer nucleotide sequences containing 96 and 128 degeneracies, corresponding to two overlapping amino acid sequences which constitute part of the sequences to which the 26-base probes correspond (Fig. 1Ac and d). An oligonucleotide probe corresponding to a sequence located close to the 5' terminus of the longest of the partial cDNA clones isolated with the degenerated probes (nucleotides 478-458 in Fig. 1D) was applied for further screening cDNA libraries for a full length cDNA clone. ^{32}P-labeling of the probes, using T4 polynucleotide kinase, plating of the phages in lawns of bacteria, their screening with the radio-labelled probes, isolation of the positive clones and subcloning of their cDNA inserts were carried out using standard procedures (Sambrook, J., et al., (1989) Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press).

c) Nucleotide sequencing of the cDNA clones

cDNA inserts isolated from positive lambda GT11 recombinant phages were subcloned into the pBluescript KS(-) vector. Inserts found in lambda ZAP phages were rescued by excising the plasmid pBluescript SK(-) in them, using the R408 helper phage (Short, J.M., et al., (1988) Nucl.Acids Res. 16, pp. 7583-7600). DNA sequencing in both directions was done by the dideoxy chain termination method. Overlapping deletion clones of the cDNAs were generated, in both orientations, by digestion of the cDNA with exonuclease III ("Erase a base" kit, Promega Biotec, Madison, WI). Single stranded templates d riv d

from these clones using the R408 phage ware sequenced with a T7 DNA polymerase sequencing system (Promega).

d) Constitutive expression of the type I human TNF-R in CHO cells

The E13 insert was introduced into a modified version of the pSVL expression vector. This construct was transfected, together with the pSV2-DHFR plasmid which contains the DHFR cDNA, into DHFR deficient CHO cells, using the calcium phosphate precipitation method. Transfection with a recombinant pSVL vector which contained the E13 insert in the inverse orientation served as a control. Cells expressing the DHFR gene were selected by growth in nucleotide-free MEM alpha medium containing fatal calf serum which had been dialyzed against phosphate buffered saline. Individual clones were picked out and then further selected for amplification of the transfected cDNAs by growth in the presence of 500 nM sodium methotrexate.

e) Detection of surface-expressed type I TNF-R in the CHO cells

Binding of radiolabelled human rTNF to cells (seeded in 15 mm tissue culture plates at a density of 2.5 X 10^5 cells/plate) was quantitated as described before (Holtmann, H. and Wallach, D. (1987) J.Immunol. 139, pp. 1161-1167).

To examine the binding of monoclonal antibodies against TBP-I to CHO cells, the cells were detached by incubation in phosphate buffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 m KCl, 0.5 m $MgCl_2$, 0.9 m $CaCl_2$), containing 5 mM EDTA and then incubated for 45 min. at 0 °C with 50 µg/ml of the test monoclonal antibody in PBS containing 0.5% bovine serum albumin, and 15 mM sodium azide (PBS/BSA). After washing the cells with PBS/BSA they were incubated further for 30 min. at 0 °C with FITC labelled, affinity purified goat antibody to the F(ab) fragment of mouse IgG (1:20 in PBS/BSA) (Bio-Makor, Israel) and then analyzed by determining the intensity of fluorescence in samples of 10^4 cells using the Becton Dickinson fluorencence activated cell sorter 440. Three monoclonal antibodies to TBP-I, clones 17,18 and 20, shown by cross competition analysis to recognize three spatially distinct epitopes in the TBP-I molecule (European Patent Application No. 90115105.0) and, as a control, a monoclonal antibody against TNF-α (all purified from ascitic fluids by ammonium sulphate precipitation and of the IgG2 isotype), were used.

f) Quantitation of the soluble form of the type I TNF-R by ELISA

A sensitive enzyme linked immunosorbent assay was set up using TBP-I-specific monoclonal and polyclonal antibodies in a sandwich technique. Immunoglobulins of the anti-TBP-I mAb clone 20 (European Patent Application No. 90115105.0) were adsorbed to 96-well ELISA plates (maxisorp, Nunc, Denmark) by incubation of the plates for 2 h. at 37 °C with a solution of 25 µg/ml of the antibody in PBS. After incubating the wells further for 2 h. at 37 °C with a solution containing phosphate buffered saline, 1% BSA, 0.02% NaN_3 and 0.05% Tween 20 (blocking solution) to block nonspecific further binding of protein, tested samples were applied in aliquots of 50 µl/well. The plates were then incubated for 2 h. at 37 °C, rinsed 3 times with PBS supplemented with 0.05% Tween 20 (washing solution) and rabbit polyclonal antiserum against TBP-I, diluted 1:500 in blocking solution, was added to the wells. After further incubation for 12 h. at 4 °C the plates were rinsed again and incubated for 2 h. with horse raddish peroxidase-conjugated purified goat anti-rabbit IgG. The assay was developed using 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 600 nm. Pure TBP-I served as a standard.

g) Detection of a soluble form of the type I TNF-R in the growth medium of the transfected CHO cells and its analysis by reversed phase HPLC

The amounts of the soluble form of the type I TNF-R in samples of the medium of the tested CHO cells, collected 48 h after medium replacement, were determined by the immunoassay described above. For analysis of the soluble receptor by rev rsed phase HPLC the CHO cells were cultured for 48 h. in serum-free medium (nucleotide-free MEM α). The medium samples were concentrated 100-fold by ultrafiltration on an Amicon PM5 membrane and 100 µl aliquots were then applied to an Aquapore RP300 column (4.5 X 30 mm, Brownlee Labs) preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with this solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then

eluted with a gradient of acetonitrile conc ntration in 0.3% aqueous trifluoroacetic acid, as described before (Engelmann, H., et al., (1989) J.Biol.Chem. 264, pp. 11974-11980). Fractions of 0.5 ml were collected and, after concentration in vacuo, were neutralized with 1 M HEPES buffer pH 9.0. Amounts of soluble type I TNF-R in the fractions were determined by ELISA and the concentration of protein by the fluorescamine method.

EXAMPLE 2

a) Cloning of the cDNA for the Type I TNF-R

To clone the cDNAs which code for the TNF-binding protein, TBP-I, and its related TNF receptor, several cDNA libraries were screened, using 3 overlapping oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBP-I (Fig. 1A). In a lambda GT11 library derived from the mRNA of human colon (randomly primed, Clontech, Palo Alto, CA), four recombinant phages which hybridized with the three probes were detected. The inserts in these four phages were similar in size, and were found to overlap by restriction mopping and sequence analysis.

Complete analysis of the sequence of the longest of the four (C2 in Fig. 1B, deposited on 6.12.1989 with the Collection Nationale de Cultures de Microorganismes (C.N.C.M.), Paris, France, Accession No. I-917) revealed an open reading frame, extended over its entire length. A polypeptide chain encoded in this reading frame fully matches the NH_2-terminal amino acid sequence of TBP-I. Neither an initiation nor a stop codon was found in the C2 insert. Rescreening the colon cDNA library, using another probe corresponding to a sequence found in C2 (see Example 1: Procedures), yielded several other recombinant phages containing inserts that overlap with the C2 insert. However, none of them provided further sequence information on the cDNA in the 5' or the 3' direction. In a lambda ZAP cDNA library derived from the mRNA of CEM lymphocyte: (Oligo dT and randomly primed, Clontech) five phages hybridizing with this probe were detected, which contained significantly longer inserts than C2.

The longest insert (E13, Fig. 1B) was sequenced in its entirety (Fig. 1D) and was found to contain the C2 sequence (nucleotides 346-1277 in Fig. 1D) within one long open reading frame of 1365 bp, flanked by untranslated regions of 255 and 555 nucleotides at its 5' and 3' ends, respectively. The potential ATG initiation site, occurring at positions 256-258 in the nucleotide sequence (denoted by an asterisk in Fig. 1D) is preceded by an upstream in-frame termination codon at bases 244-246. The start location is in comformity with one of the possible alternatives for the translation initiation consensus sequence (GGCATGG, nucleotides 253-259).

There is no characteristic poly(A) addition signal near the 3' end of the cDNA. The sequence ACTAAA, at nucleotides 2045-2050, may serve as an alternative to this signal, but with low efficiency. At nucleotides 1965-2000, there are six consecutive repeats of the sequence G(T)n (n varying between 4 and 8).

The size of the protein encoded by the cDNA (about 50 kD) is significantly larger than that of TBP-I. A hydropathy index computation of the deduced amino acid sequence of the protein (Fig. 1C) revealed two major hydrophobic regions (see round-ended boxes in Fig. 1D). One, at its NH_2-terminus, is apparently the signal peptide whose most likely cleavage site lies between the glycine and isoleucine residues designated in Fig. 1D as -1 and +1 respectively. The other major hydrophobic domain, located between residues 191 and 213, is flanked at both ends by several charged amino acids, characteristic of a membrane anchoring domain. As in several other transmembrane proteins, the amino acids confining the hydrophobic domain at its COOH-terminal are basic. The transmembrane domain bisects the predicted protein into almost equally sized extracellular and intracellular domains.

The extracellular domain has 3 putative sites for asparagine-linked glycosylation (overlined in Fig. 1D). Assuming that the amount of oligosaccharides in the extracellular domain is similar to that reported as present in TBP-I (Seckinger, P., et al., (1989b) Cytokine I, 149 (an abstract)), the molecular size of the mature protein is very similar to that estimated for the type I receptor (about 58kD) (Hohmann, H.P., et al., (1989) J.Biol.Chem. 264, pp. 14927-14934).

b) Features of the predicted amino acid sequence in the Type I TNF-R and relationship to the structure of TBP-I and TBP-II

The amino acid sequence of the extracellular domain of the protein encoded by the E13 cDNA fully matches several sequences of amino acids determined in TBP-I (Table I). It contains the NH_2-terminal amino acid sequenc of TBP-I at amino acids 20-32 (compare Fig. 1D and Fig. 1Aa), a sequence corresponding to the COOH terminus of TBP-I at amino acid 178-180; and, also, adjacent to the first

methionine located further downstr am in the ncoded protein, a sequence which is identical to the NH_2-terminal amino acid sequence of a cyanog nbromide cleavage fragment of TBP-I (broken lines in Fig. 1D). There is also a marked similarity in amino acid composition between the extracellular domain of the receptor and TBP-I (Table II).

The most salient feature of this amino acid composition is a very high content of cystein residues (shown boxed in Fig. 1D). The positioning of the cystein residues as well as of other amino acids within the extracellular domain displays a four-fold repetition pattern (underlined in Fig. 1D). The amino acid sequence within the extracellular domain of the TNF-R, which corresponds to the COOH terminal sequence of TBP-I (see Table I and Fig. 4), is located at the COOH terminus of the cystein-rich repeat region. The sequence corresponding to the NH_2 terminal sequence of TBP-I corresponds to a sequence located a few amino acids upstream of the NH_2 terminal end of this region (broken lines in Fig. 1D) in the extracellular domain.

In contrast to the identity of amino acid sequences between TBP-I and the extracellular domain of the type I TNF receptor, sequences examined in the soluble form of the type II TNF-R (TBP-II, Table I) were not identical to any sequence in the type I TNF-R. This finding is expected, considering the lack of immunological crossreactivity between the two receptors (Engelmann, H., et al., (1990) J.Biol.Chem. 265, pp. 1531-1536).

In contrast to the very high content of cystein residues in the putative extracelluiar domain of the type I TNF-R, there are only 5 cystein residues in the intracellular domain. Between the two which are proximal to the transmembrane domain (positions 227 and 283), extends a stretch of 55 amino acids which is rich in proline residues (15% of the residues) and even richer in serine and threonine residues (36%), most located very close to or adjacent to each other.

EXAMPLE 3

Expression of the type I TNF-R cDNA

To explore the relation between the protein encoded by the E13 cDNA and TBP-I further, this protein was expressed in CHO cells. The E13 cDNA was introduced into an expression vector and was cotransfected with a recombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient cells. After selection by growth in a nucleotide-free medium, individual clones were amplified by growth in the presence of methotrexate. A number of clones which react with several monoclonal antibodies that bind to spatially distinct epitopes in TBP-I were detected (Fig.2). Expression of the protein was correlated with an increase in specific binding of human TNF to the cells (Table III).

Applying a sensitive immunoassay for TBP-I in which polyclonal antibodies and a monoclonal antibody against this protein were employed, (Procedures, Example 1f) in the medium of CHO cells which express the human TNF-R on their surface, also a soluble form of the protein could be detected (Table III). All of five different CHO clones which expressed the TNF-R, produced this soluble protein. Several other transfected clones which did not express the cell surface receptor did not produce its soluble form either. When analyzed by reversed phase HPLC, the CHO-produced soluble TNF-R eluted as a single peak, with a retention time identical to that of TBP-I (Fig. 3).

EXAMPLE 4

Cloning of the cDNA encoding TBP-I and expression of TBP-I in Chinese Hamster Ovary (CHO) cells

In order to obtain plasmids suitable for efficient expression of the DNA encoding a soluble domain of the type I TNF receptor in mammalian cells, the gene from position 256 to position 858 of the DNA sequence shown in Fig. 1D, was cloned in two expression vectors: in one plasmid, gene expression was under the SV40 early gene promoter; in the second plasmid, gene expression was under the regulation of the cytomegalovirus (CMV) promoter. These vectors were introduced into CHO cells by $CaPO_4$ coprecipitation with a plasmid DHFR selectable genetic marker.

Construction of Expression Vectors

1) SV40 Early Promoter-TBP-I fusion: Plasmid pSV-TBP.

Constitutive expression of TBP-I can b achieved by using an expression vector that contains the DNA sequence coding for TBP-I fused to th strong SV40 early gene promoter (Fig 5).

Step 1: A DNA fragment coding for TBP-I, including its signal peptide and xtending to amino acid 180 was prepared by PCR amplification. For amplification two oligonucleotides were used as primers: the 5' end primer contains the sequence coding for the first seven amino acids of the signal peptide, preceded by six nucleotides; the 3' end oligonucleotide contains the sequence coding for amino acid residues 174 through 180 followed by two stop codons (TGA and TAA).

The conditions for PCR amplification are the following:

	Temperature °C	Time min
1 cycle	94 50 72	6 2 4
30 cycles	94 50 72	1 2 4
1 cycle	94 50 72	1 2 12

Step 2: After sequence verification, the amplified DNA fragment was cloned into the HincII restriction site of plasmid pGEM-1 by blunt end ligation. Plasmids pTBP-16 and pTBP-17 are the two plasmids obtained in this way and they differ in the orientation of the TBP-I insert with respect to the cloning vector.

Step 3: The DNA fragment containing TBP-I was excised from plasmid pTBP-17 using the two adjacent restriction sites HindIII (at the 5' end) and BamHI (at the 3' end).

Step 4: Finally, this fragment was cloned between the HindIII and the BclI restriction sites of the expression vector pSVE3.

The resulting plasmid is called pSV-TBP (Fig. 5).

2) CMV promoter-TBP-I fusion: plasmid pCMV-TBP.

Alternatively, constitutive expression of TBP-I can be achieved by using an expression vector that contains the DNA sequence coding for TBP-I fused to the CMV promoter (Fig 6).

The first two steps for the construction of the CMV based vector are identical to the ones described for the construction of the SV40-TBPI fusion plasmid, as described above.

Step 3: The DNA fragment containing TBP-I was excised from plasmid pTBP-17 using the two adjacent restriction sites HindIII (at the 5' end) and XbaI (at the 3' end).

Step 4: Finally, this fragment was cloned between the HindIII and the XbaI restriction sites of the expression vector Rc/CMV.

The resulting plasmid is called pCMV-TBP.

Expression of Human TBP-I in CHO Cells

CHO cells CHO-K1 DHFR$^-$, lacking DHFR activity, were transformed by $CaPO_4$ coprecipitation with a 12:1 mixture of uncut pSV-TBP DNA (73 µg) and mpSV2DHFR (6µg) DNA, the latter being the selectable marker. Alternatively, CHO cells were transformed with a 5:1 mixture of pCMV-TBP (30 µg) and mpSV2DHFR (6 µg).

Cells were grown in nutrient mixture F12 (Gibco) with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. For DNA transfection, $5x10^5$ cells were cultured for one day in 9 cm plates. A $CaPO_4$-DNA coprecipitate was prepared by mixing plasmid DNAs, dissolved in 0.45 ml of Tris-HCl pH 7.9, 0.1 mM EDTA with 0.05 ml of 2.5 M $CaCl_2$; thereafter, 0.5 ml of 280 mM Na_2PO_4, 50 mM Hepes buffer pH 7.1 was added with gentle mixing. The mixture was kept for 30-40 minutes at room temperature in order to form the precipitate. After adding the $CaPO_4$-DNA to the cells and leaving the cells at room temperature for 30 min, 9 ml of nutrient mixture F12, 10% FCS were added and the cultures returned to the CO_2 incubator for 4 hours. Medium was removed and the cells were osmotically shocked with 10% glycerol in F12 for 4 min. After 48 hours of growth in non-selective medium, the cells were then trypsinized and subcultured 1:10 into selectiv

medium, composed of Dulbecco's modified Eagle's medium (DMEM) (H21, Gibco), 150 µg/ml proline, and 10% FCS which had been extensively dialyzed against phosphate-buffered saline (PBS). In some cases, MEM alpha medium without nucleotides (F20, Gibco) was used. The cultures were kept at 37 °C and 10% CO_2 and the medium was changed every 3-4 days. Clones were isolated after about 15 days, trypsinized, and grown to mass cultures.

Transformants able to grow in medium lacking thymidine (DMEM with dialyzed serum) were obtained. Culture supernatants of individual transformant clones or culture supernatant of mixed populations were screened for human TBP-I by measuring the level of secreted protein by the enzyme linked immunoassay described in Example 1f. TBP-I levels of up to 10 ng/ml ware detected in culture supernatants of mixed cells populations.

This example shows that TBP-I or a similar soluble protein can be obtained also by transfection of mammalian cells with a DNA encoding the soluble domain of the type I TNF receptor.

EXAMPLE 5

Expression of TBP-I in E. coli.

For expression of TBP-I in E.coli, the sequence coding for the signal peptide and for the first 19 aminoacids (Arg) must be removed (Figure 1D). Moreover, the Asp residue must be preceded by a Met residue. The desired fragment is then cloned into the expression hector pKK223-3 that contains the hybrid tryp-lac promoter. To achieve this goal plasmid pTBP-16 (Fig 5) is cut with the two unique restriction sites StyI and HindIII. StyI restriction site is C/CAAGG and, therefore, it cuts after Pro24. HindIII restriction site is located in the polylinker of the plasmid and downstream from the two added stop codons that follow Asn180 (Fig. 5).

The resulting DNA fragment, coding for TBP-I, has an intact 3' end and a truncated 5' end, where the sequence preceding the StyI site and coding for Asp-Ser-Val-Cys-Pro has been removed.

For cloning of the StyI-HindIII fragment into the expression vector pKK223-3, the following couple of synthetic oligonucleotides are used:

```
                   Met Asp Ser Val Cys Pro
   5'   AATTC      ATG GAT AGT GTG TGT CCC          3'
   3'          G   TAC CTA TCA CAC ACA GGG GTT C    5'
         EcoRI                            StyI
```

One end of this double stranded oligonucleotide is an EcoRI restriction site. This end is ligated to the EcoRI site of plasmid pKK223-3, located downstream to the tryp-lac promoter. The second end of the double stranded oligonucleotide is a StyI restriction site to be ligated to the StyI of the TBP-I DNA fragment.

The remainder of the sequence is such that the codons coding for the first five amino acids are restored and that an additional Met codon is added in front of Asp20. The expression vector is obtained by ligation of plasmid pKK223-3, digested with EcoRI and HindIII, to the double-stranded synthetic oligonucleotide and to the StyI-HindIII TBPI fragment.

E.coli cells are transfected with this expression vector in order to produce TBP-I.

Table I: Amino acid sequences of TBP I and TBP II

TBP I:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
CNBr-1 (=N-terminus)	NH_2	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	ILe	His	Pro	Gln	---		
CNBr-2	NH_2	Gly	Gln	Val	Glu	ILe	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	---
C-terminus	---	ILe	Glu	Asn	COOH												

TBP II:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
N-terminus	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	Tyr	---
TRP 35	NH_2	Leu	Cys	Ala	Pro	Leu	Arg	Lys																
TRP 39/1	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 39/2	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---											
TRP 44/1	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	---										
TRP 44/2	NH_2	Ser	Cys	Gly	Pro	Ser	Tyr	Pro	Asp	---														
TRP 46/1	NH_2	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg										
TRP 46/2	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 50	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 54/1	NH_2	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys														
TRP 54/2	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg						
TRP 53/1	NH_2	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg								
TRP 53/2	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 60	NH_2	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	---									
TRP 62	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys			
TRP 65	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	Lys			
TRP 67	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys			
TRP 84	NH_2	Thr	Ser	Asp	Thr	Val	Cys	Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	---				

Table II

Similarity of the amino acid compositions of the TNF binding protein TBPI and a corresponding region in the extracellular domain of the TNF−R (type I)		
Amino acid	mol/100 mol of amino acids	
	TBPI*	Residues 20-180 in the extracellular domain**
Ala 1.7	1.2	
Cys	12.8	14.9
Asp + Asn	10.9	11.1
Glu + Gln	13.9	12.4
Phe	3.2	3.1
Gly	6.3	5.6
His	4.4	4.3
Ile	2.8	2.5
Lys	6.2	6.2
Leu	8.0	6.8
Met	0.4	0.6
Pro	3.8	3.1
Arg	4.7	4.3
Ser	8.1	9.3
Thr	6.1	6.2
Val	4.2	4.3
Trp	-	0.6
Tyr	2.4	3.1

* According to Olsson et al., 1989

**Residue 20 corresponds to the NH_2-terminal amino acid of TBPI. Residue 180 is the COOH-terminal residue of TBPI.

Table III

Expression of the cell surface and soluble forms of human type I TNF−R in CHO cells

CHO cell clone	Specific binding of TNF (CPM/10^6 cells)	cells expressing human cell surface TNF-R (% fluorescent cells)	human soluble type I TNF receptors (pg/ml)
nontransfected	180±45	<1%	<0.03
C6	175±50	<1%	<0.03
R-16	550±60	73%	30
R-18	610±40	89%	49

The R-16 and R-18 clones consist of cells transfected with a recombinant expression vector containing E13 cDNA. C-6 cells were transfected with a control vector. Binding of radiolabelled TNF to the cells was determined in pentaplicate samples. Detection of immunoreactive receptors on the surface of the cells was carried out using combined 17, 18, 20 and 30 anti TBPI monoclonal antibodies. Results are expressed as percentage of fluorescent cells (background values, obtained by staining the cells with an anti-TNF monoclonal antibody, are subtracted).

Claims

1. A method for the production of human TBP-I (Tumor Necrosis Factor Binding Protein I = soluble Tumor Necrosis Factor Receptor type I (sTNF-RI)) which comprises:
   (a) transfecting eukaryotic cells with an expression vector comprising a DNA molecule encoding the whole human TNF-RI;
   (b) culturing the transfected eukaryotic cells; and
   (c) isolating said TBP-I from the medium.

2. The method according to claim 1, wherein the DNA molecule encoding the whole type I TNF receptor is the cDNA having the sequence depicted in Figure 1D.

3. The method according to claim 1 or 2, wherein the cDNA is introduced into an expression vector and is cotransfected with a recombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient Chinese hamster ovary (CHO) cells.

4. The method according to any one of claims 1 to 3, wherein the cells are selected by growth in a nucleotide-free medium, individual clones are amplified by growth in the presence of methotrexate and the TBP-I secreted into the medium is detected by reaction with monoclonal and/or polyclonal antibodies raised against TBP-I.

5. The method according to any one of claims 1 to 4, wherein said TBP-I obtained is a TBP-I precursor or analog.

Patentansprüche

1. Verfahren zur Herstellung von menschlichem TBP-I (Tumor-nekrosefaktor-Bindungsprotein I = löslicher Tumornekrosefaktor-Rezeptor-Typ I (sTNF-RI)) umfassend:
   (a) Transfektion eukaryotischer Zellen mit einem Expressionsvektor, der ein den vollständigen menschlichen TNF-RI codierendes DNA-Molekül enthält;
   (b) Züchtung der transfizierten eukaryotischen Zellen; und
   (c) Isolierung des TBP-I aus dem Medium.

2. Verfahren nach Anspruch 1, wobei das den vollständigen TypI-TNF-Rezeptor codierende DNA-Molekül die cDNA mit der in Figur 1D dargestellten Sequenz ist.

3. Verfahren nach Anspruch 1 oder 2, wobei die cDNA in einen Expressionsvektor eingefügt und mit einem rekombinanten, die Dihydrofolatreduktase (DHFR)-cDNA enthaltenden Vektor in DHFR-defiziente Ovarzellen des chinesischen Hamsters (CHO-Zellen) cotransfiziert wird.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei die Zellen durch Wachstum in einem nucleotidfreien Medium selektioniert werden, einzelne Clone durch Wachstum in Gegenwart von Methotrexat amplifiziert werden und das in das Medium sezernierte TBP-I durch Reaktion mit gegen TBP-I gerichteten monoclonalen und/oder polyclonalen Antikörpern nachgewiesen wird.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das erhaltene TBP-I eine TBP-I-Vorstufe oder ein TBP-I-Analogon ist.

Revendications

1. Procédé de préparation de TBP-I humaine (Protéin I d liaison au facteur de nécrose tumorale = récepteur du facteur soluble de nécrose tumorale du type I (sTNF-RI)), comprenant :
   (a) la transfection de cellules eucaryotes avec un vecteur d'expression comprenant une molécule d'ADN codant pour l'ensemble du TNF-RI humain ;
   (b) la mise en culture des cellules eucaryotes de transfection ; et
   (c) l'isolement dudit TBP-I du milieu.

2. Procédé selon la revendication 1, dans lequel la molécule d'ADN codant pour l'ensemble du récepteur de TNF du type I est l'ADNc ayant la séquence représentée sur la figure 1D.

3. Procédé selon la revendication 1 ou 2, dans lequel l'ADNc est introduit dans un vecteur d'expression et soumis à une cotransfection avec un vecteur recombinant contenant l'ADNc de dihydrofolate-réductase (DHFR) pour former des cellules d'ovaire de hamster chinois (CHO) présentant une carence en DHFR.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel les cellules sont sélectionnées par croissance dans un milieu exempt de nucléotides, des clones individuels sont amplifiés par croissance en présence de méthotrexate, et la TBP-I sécrétée dans le milieu est détectée au moyen d'une réaction avec des anticorps monoclonaux et/ou des anticorps polyclonaux dirigés contre la TBP-I.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel ladite TBP-I obtenue est un précurseur ou un analogue de la TBP-I.

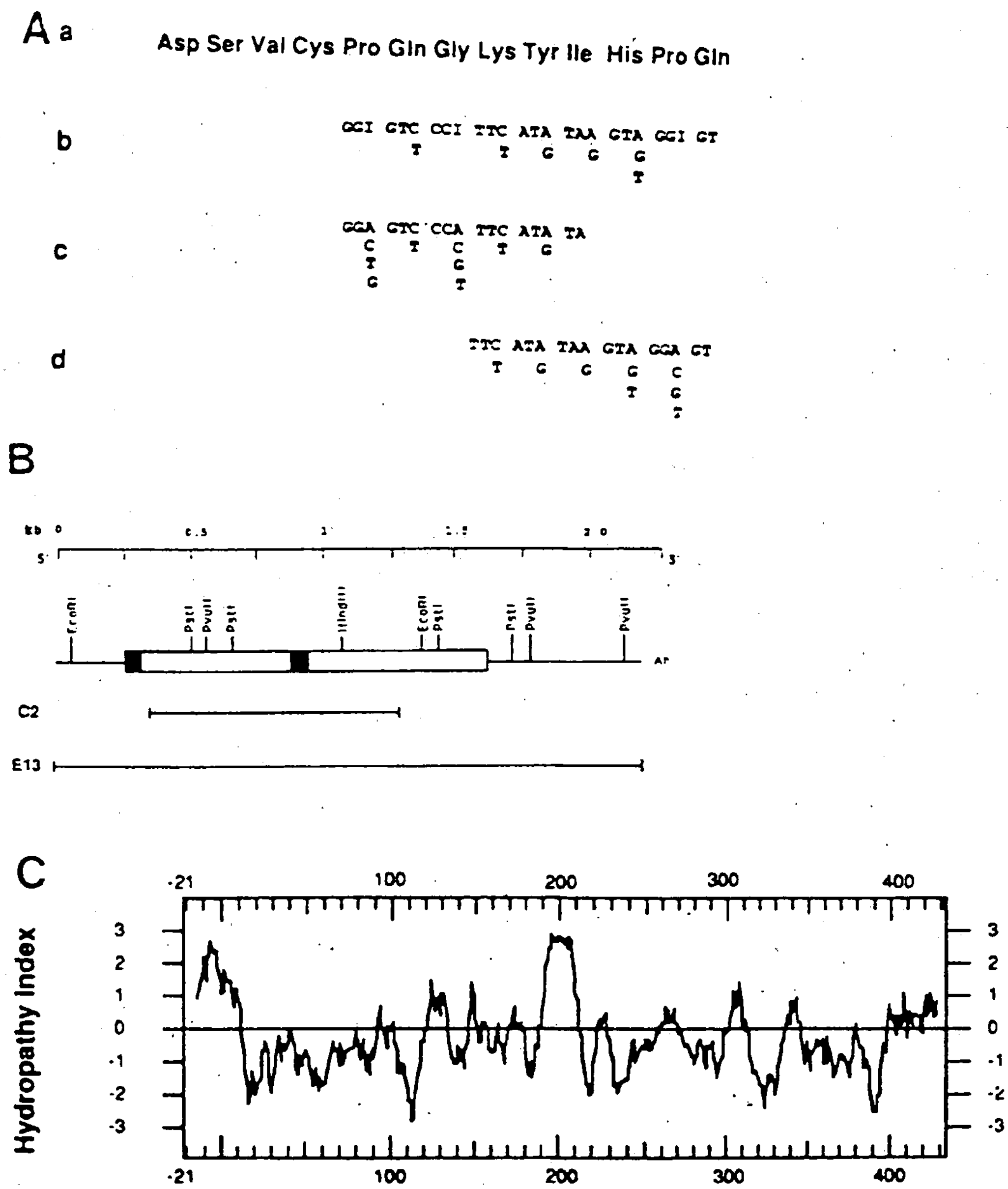
A
a
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln
b
GGI GTC CCI TTC ATA TAA GTA GGI GT
c
GGA GTC CCA TTC ATA TA
d
TTC ATA TAA GTA GGA GT
B
kb 0
5'
3'
C2
E13
C
-21
100
200
300
400
Hydropathy Index
3
2
1
0
-1
-2
-3

Figure 1 A-C

D

1 CGGCCCAGTGATCTTGA 17

18 ACCCCAAAGGCCAGAACTGGAGCCTCAGTCCAGAGAATTCTGAGAAAATTAAAGCAGAGAGGAGGGGAGAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAA 136

137 CTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTGTCTGGC 255

-21 -10 -1 +1

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu

256 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG 345

*

10 20 30

Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr

346 GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC 435

40 50 60

Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr

436 AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC 525

70 80 90

Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp

526 GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC 615

100 110 120

Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu

616 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC 705

130 140 150

Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val

706 AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC 795

160 170 180

Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr

796 TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC 885

190 200 210

Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys

886 ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG 975

Figure 1 D (part 1)

```
     220                                           230                                           240
     Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
 976 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC 1065
     250                                           260                                           270
     Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
1066 CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC 1155
     280                                           290                                           300
     Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
1156 CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC 1245
     310                                           320                                           330
     Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
1246 TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC 1335
     340                                           350                                           360
     Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu Glu Leu
1336 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG ATC GAT CGG CTG GAG CTG 1425
     370                                           380                                           390
     Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu Glu Leu
1426 CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG 1515
     400                                           410                                           420
     Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala
1516 CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCC CTC CCG CCC GCG 1605
```

Figure 1 D (part 2)

```
         430
         Pro Ser Leu Leu Arg End
1606     CCC AGT CTT CTC AGA TGA GGCTGCGCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTCTGGAAAGGAGGGGTCCTGCAGGGGCAAGC   1
                              *
1719     AGGAGCTAGCAGCCGCCTACTTGGTGCTAACCCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCGCGCGGAGAGAGGTGCGCCGTGGGCTCAA   1
1838     GAGCCTGAGTGGGTGGTTTGCGAGGATGAGGGACGCTATGCCTCATGCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTGGTTCGTCCCTGAGCCTTTTTCACAGTG   1
1957     CATAAGCAGTTTTTTTTTGTTTTTGTTTTGTTTTGTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTGCCTGGACAAGCACATAGCAAGCTGAA   2
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Figure 1 D (part 3)

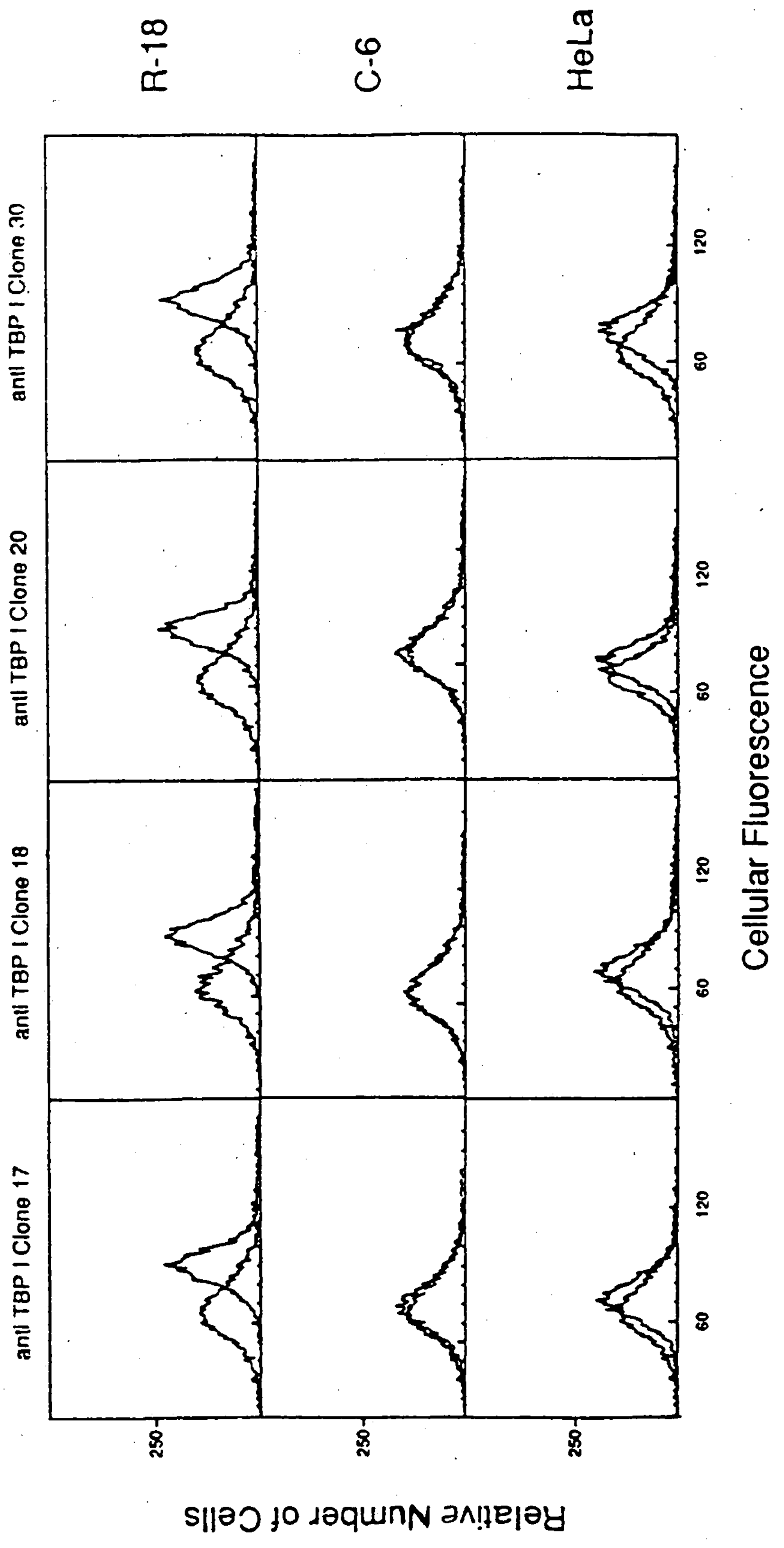
anti TBP I Clone 17
anti TBP I Clone 18
anti TBP I Clone 20
anti TBP I Clone 30
R-18
C-6
HeLa
250
60
120
Cellular Fluorescence
Relative Number of Cells

Figure 2

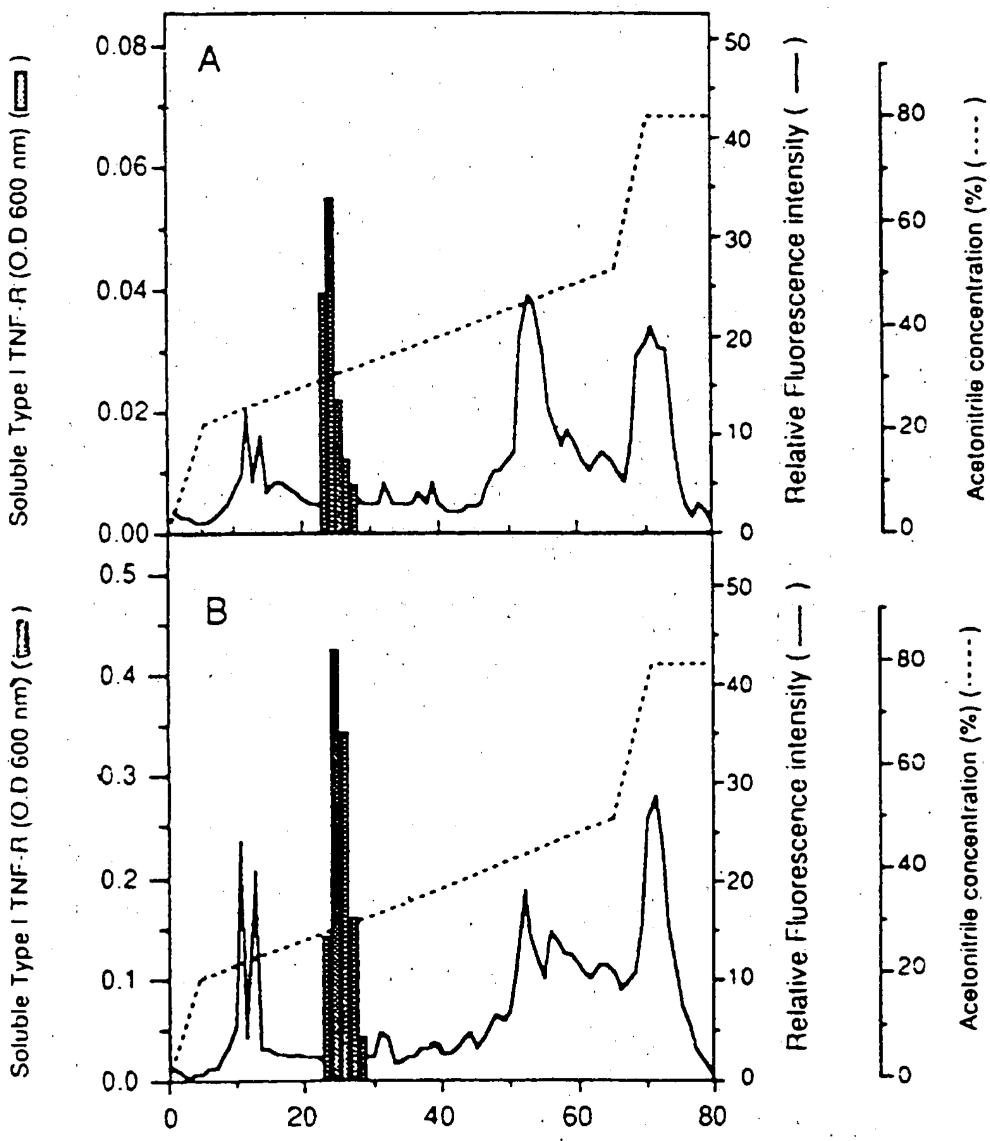
A
B
Soluble Type I TNF-R (O.D 600 nm)
Relative Fluorescence intensity (—)
Acetonitrile concentration (%) (----)
0.00
0.02
0.04
0.06
0.08
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
0
20
40
60
80
0
20
40
60
80

Figure 3

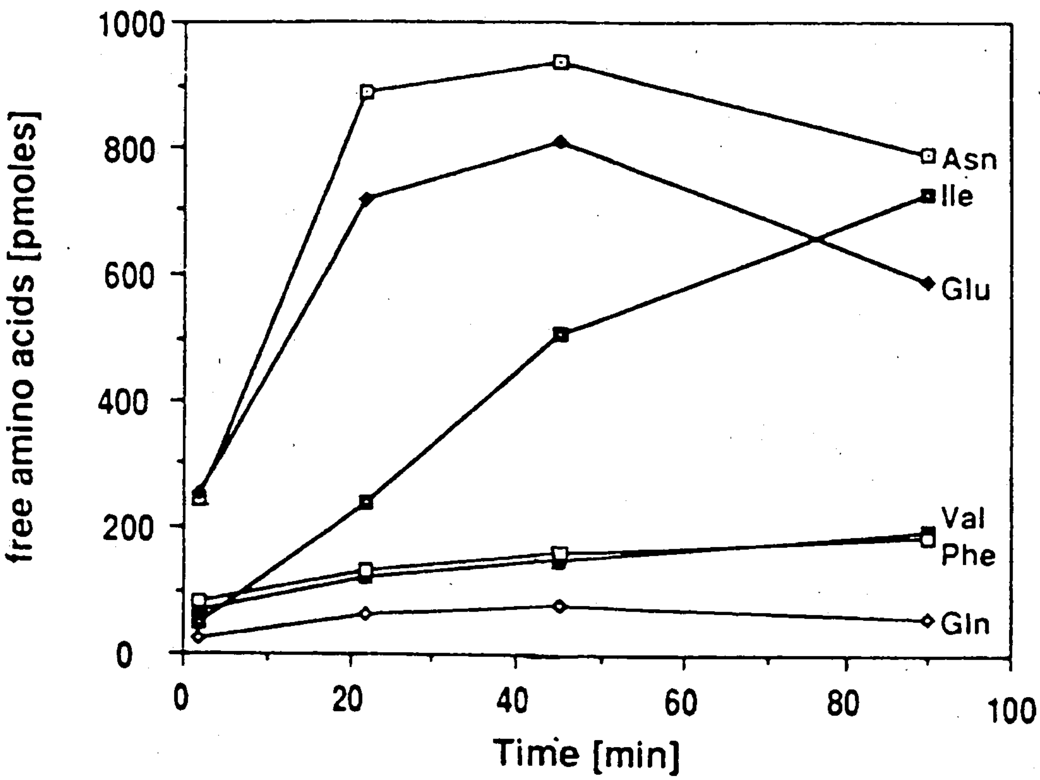
1000
800
600
400
200
0
free amino acids [pmoles]
0
20
40
60
80
100
Time [min]
Asn
Ile
Glu
Val
Phe
Gln

Figure 4

CONSTRUCTION OF PLASMID pSV-TBP

TNF RECEPTOR cDNA

```
              -21                                                         180
              Met                                                         Asn
5'············ATG GGC CTC TCC ACC GTG CCT·····TGC CTA CCC CAG ATT GAG AAT·········3'
3'············TAC CCG GAG AGG TGG CAC GGA·····ACG GAT GGG GTC TAA CTC TTA·········5'
```

PCR Amplification

Phosphorilated 5' primer

```
5' CGG CCG ATG GGC CTC TCC ACC GTG CCT·······································3'
3' ········································ACG GAT GGG GTC TAA CTC TTA TGA TAA 5'
```

Phosphorilated 3' primer

615 bp fragment
(coding for TBP I including its signal peptide)

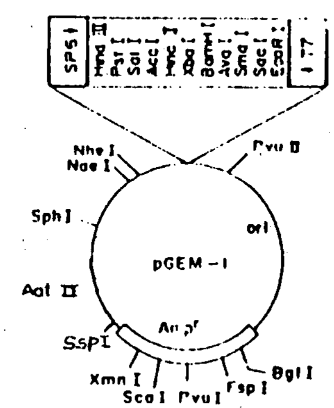

Figure 5 part 1)

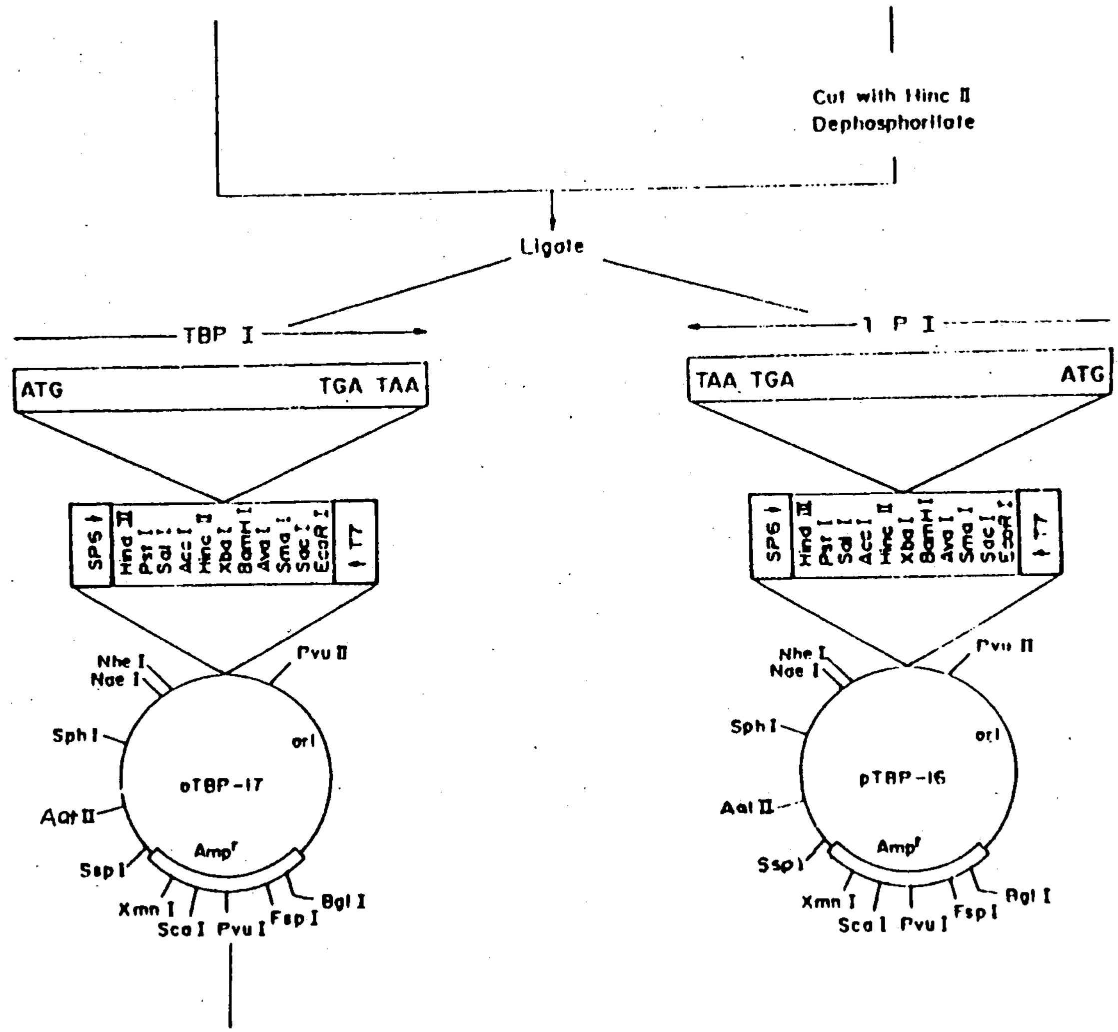

Figure 5 (part 2)

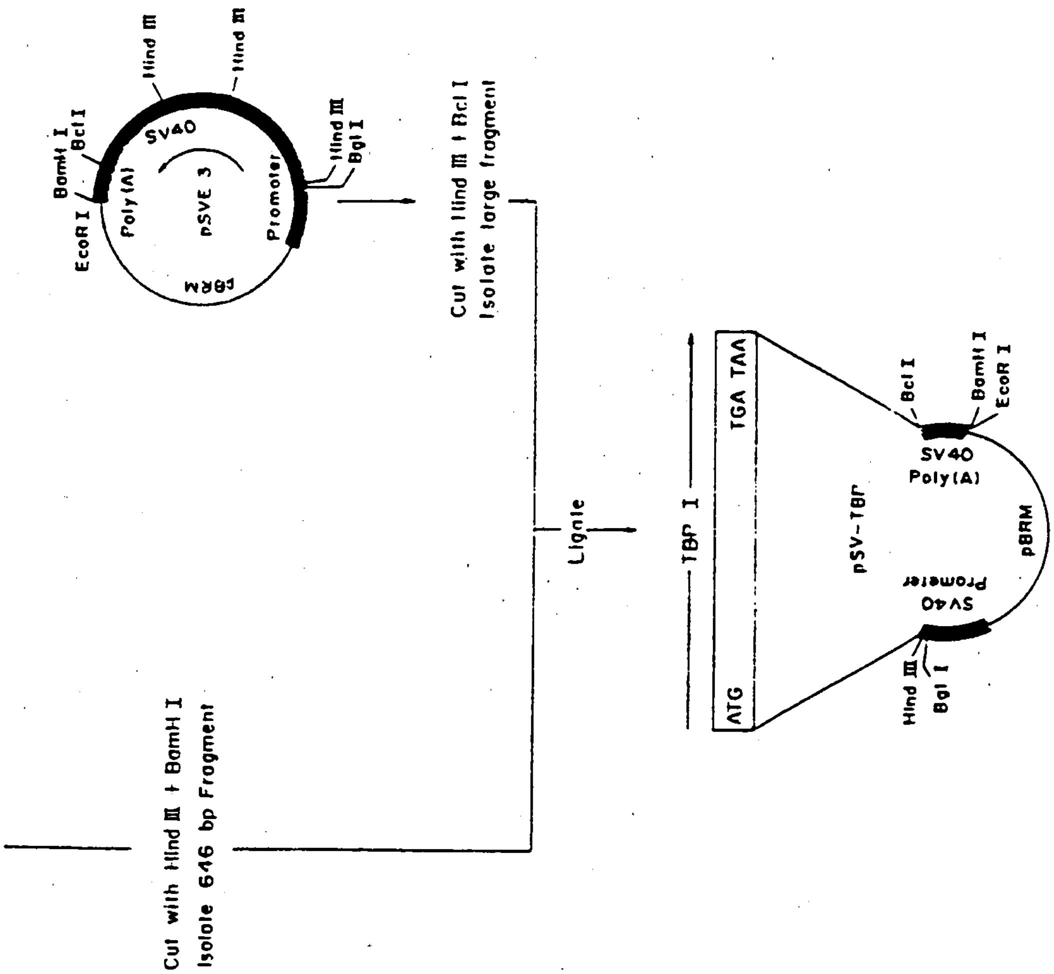

Figure 5 (part 3)

CONSTRUCTION OF PLASMID pCMV-TBP

TNF RECEPTOR cDNA

```
            -21                                                   180
            Met                                                   Asn
5'..........ATG GGC CTC TCC ACC GTG CCT      ..TGC CTA CCC CAG ATT GAG AAT.........3'
3'.     .....TAC CCG GAG AGG TGG CAC GGA.....ACG GAT GGG GTC TAA CTC TTA.........5'
```

PCR Amplification

Phosphorilated 5' primer

```
5' CGG CCG ATG GGC CTC TCC ACC GTG CCT.......................................3'
3'............................................ ACG GAT GGG GTC TAA CTC TTA TGA TAA 5'
```

Phosphorilated 3' primer

615 bp fragment
(coding for TBP I including its signal peptide)

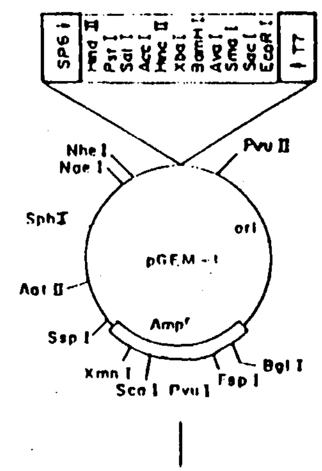

Figure 6 (part 1)

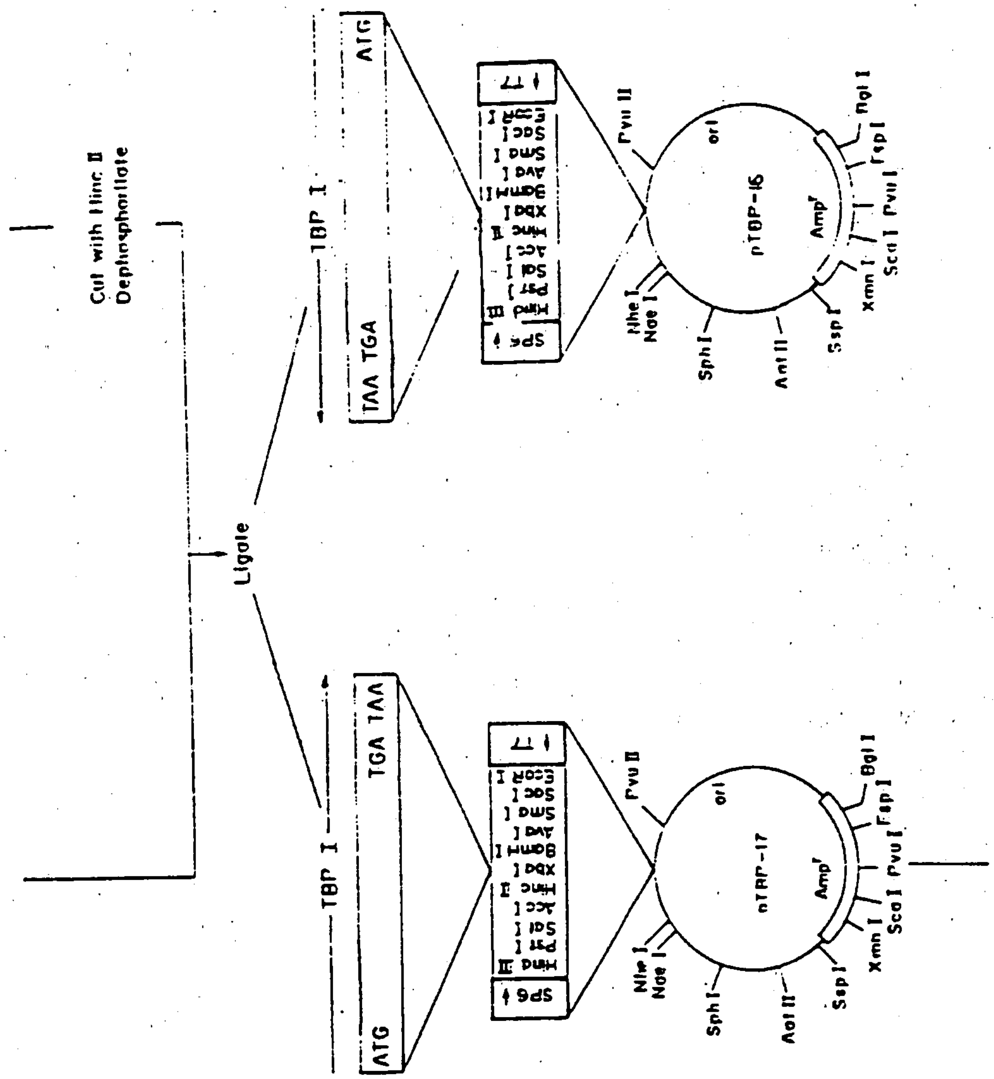

Figure 6 (part 2)

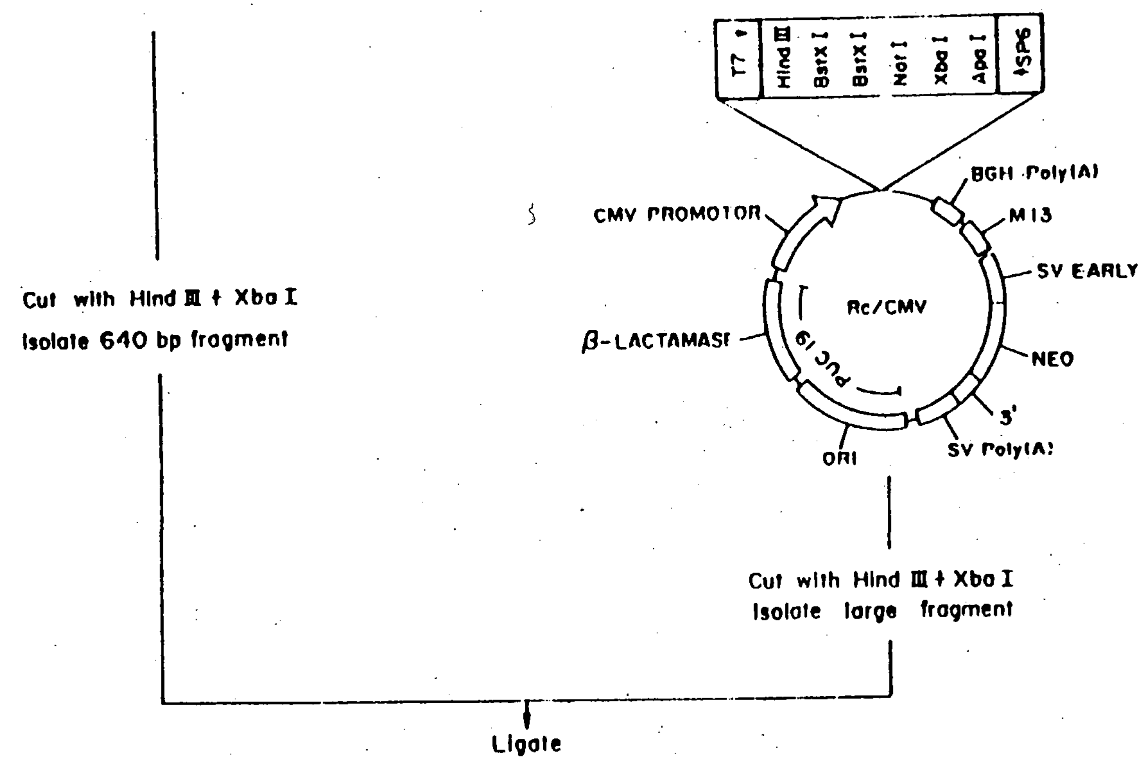

Figure 6 (part 3)

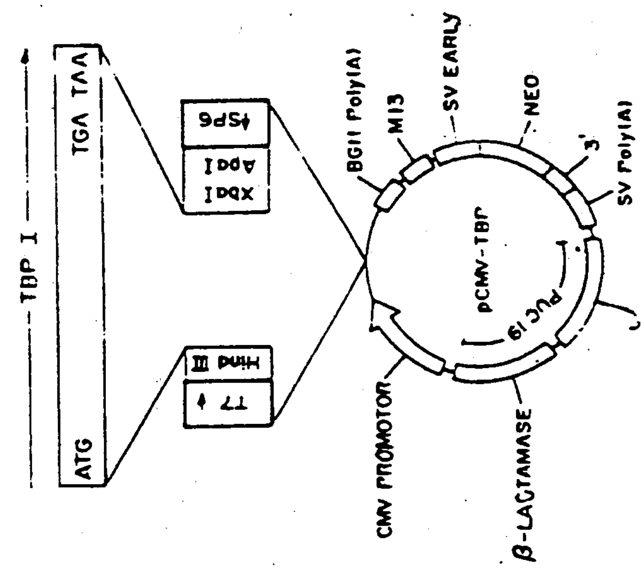

Figure 6 (part 4)